Journal of Food Protection, Vol. 68, No. 12, 2005, Pages 2594–2605

Predictive Model for *Clostridium perfringens* Growth in Roast Beef during Cooling and Inhibition of Spore Germination and Outgrowth by Organic Acid Salts†‡

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MS 05-111: Received 16 March 2005/Accepted 21 June 2005

ABSTRACT

Spores of foodborne pathogens can survive traditional thermal processing schedules used in the manufacturing of processed meat products. Heat-activated spores can germinate and grow to hazardous levels when these products are improperly chilled. Germination and outgrowth of *Clostridium perfringens* spores in roast beef during chilling was studied following simulated cooling schedules normally used in the processed-meat industry. Inhibitory effects of organic acid salts on germination and outgrowth of *C. perfringens* spores during chilling and the survival of vegetative cells and spores under abusive refrigerated storage was also evaluated. Beef top rounds were formulated to contain a marinade (finished product concentrations: 1% salt, 0.2% potassium tetrapyrophosphate, and 0.2% starch) and then ground and mixed with antimicrobials (sodium lactate and sodium lactate plus 2.5% sodium diacetate and buffered sodium citrate and buffered sodium citrate plus 1.3% sodium diacetate). The ground product was inoculated with a three-strain cocktail of *C. perfringens* spores (NCTC 8238, NCTC 8239, and ATCC 10388), mixed, vacuum packaged, heat shocked for 20 min at 75°C, and chilled exponentially from 54.5 to 7.2°C in 9, 12, 15, 18, or 21 h. *C. perfringens* populations (total and spore) were enumerated after heat shock, during chilling, and during storage for up to 60 days at 10°C using tryptose-sulfite-cycloserine agar. *C. perfringens* spores were able to germinate and grow in roast beef (control, without any antimicrobials) from an initial population of ca. 3.1 log CFU/g by 2.00, 3.44, 4.04, 4.86, and 5.72 log CFU/g after 9, 12, 15, 18, and 21 h of exponential chilling. A predictive model was developed to describe sigmoidal *C. perfringens* growth curves during cooling of roast beef from 54.5 to 7.2°C within 9, 12, 15, 18, and 21 h. Addition of antimicrobials prevented germination and outgrowth of *C. perfringens* regardless of the chill times. *C. perfringens* spores could be recovered from samples containing organic acid salts that were stored up to 60 days at 10°C. Extension of chilling time to ≥9 h resulted in >1 log CFU/g growth of *C. perfringens* under anaerobic conditions in roast beef. Organic acid salts inhibited outgrowth of *C. perfringens* spores during chilling of roast beef when extended chill rates were followed. Although *C. perfringens* spore germination is inhibited by the antimicrobials, this inhibition may represent a hazard when such products are incorporated into new products, such as soups and chili, that do not contain these antimicrobials, thus allowing spore germination and outgrowth under conditions of temperature abuse.

Clostridium perfringens is a ubiquitous organism frequently present in a variety of foods *(11)*. Meat and poultry products have been implicated in numerous outbreaks of foodborne disease *(6)*. The Centers for Disease Control and Prevention estimates that more than 248,000 cases of foodborne illness due to *C. perfringens* infection occur annually in the United States. The most commonly incriminated foods are roast beef, turkey, and chicken and gravies, juices, and dressings containing these meats *(6, 7)*.

Major contributing factors leading to food poisoning associated with *C. perfringens* include the ability of this pathogen to form heat-resistant spores that survive commercial cooking operations and to grow very rapidly at relatively high temperatures. Germination and outgrowth of *C. perfringens* spores during cooling of thermally processed meat products has been reported extensively *(2, 4, 9, 17–21, 23, 29–31, 38)*. Predictive models for *C. perfringens* growth in some food systems are currently available in print and as electronic databases such as the U.S. Department of Agriculture (USDA) Pathogen Modeling Program and ComBase *(4, 9, 17)*. Because of the health risk associated with *C. perfringens*, some meat processors incorporate cooling regimes as critical control points in their hazard analysis critical control point plans. The USDA Food Safety and Inspection Service (FSIS) published performance standards for pathogen lethality and stabilization of meat and poultry products *(34, 36)* and provided guidelines (safe

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† Contribution from the University of Nebraska Agricultural Research Division, Lincoln, journal series 14569.
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harbors) for safe cooling of these products. The stabilization performance standards focus on the germination and outgrowth of spores during cooling. The compliance guidelines for stabilization state that processed meat and poultry products must be cooled from 54.5°C (130°F) to 26.6°C (80°F) within 1.5 h and from 26.6 to 4.4°C (40°F) within 5 h. However, equipment malfunctions or power failures may occur in commercial processing operations and cause cooling deviations, resulting in growth of *C. perfringens.* Because of this possibility, additional information is necessary on the growth characteristics of this organism at different cooling rates to evaluate the risk of spore germination and outgrowth during deviations in the cooling process.

Sodium and potassium salts of organic acids such as propionic, lactic, pyruvic, acetic, and citric acids are extensively used in meat and poultry products either as flavor enhancers or to extend the microbiological shelf stability. Sodium citrate and citric acid are generally recognized as safe (GRAS) ingredients and inhibit growth of pathogens in meat products *(19, 23, 31).* The USDA FSIS approved use of sodium or potassium salts of lactic acid and sodium diacetate in meat products as antimicrobial ingredients to control pathogens *(35).* Incorporation of these antimicrobial ingredients can provide an additional measure of safety to control *C. perfringens* when cooling deviations occur *(19, 23, 26, 31, 35).*

Although *C. perfringens* spores can survive in processed meat products during refrigerated storage, vegetative cells are more sensitive and lose viability under these conditions *(30, 32).* The surviving *C. perfringens* spores can germinate and grow rapidly when these products are reheated and held at abusive temperatures in food service settings *(7).* Information on the effects of antimicrobial ingredients on vegetative cells and spores during prolonged storage of processed meats is lacking. Knowledge of the fate of vegetative cells and spores allows manufacturers, distributors, and food service operators to incorporate additional control measures to prevent *C. perfringens* multiplication and growth to potentially hazardous levels.

The objectives of the present study were to evaluate (i) germination and outgrowth of *C. perfringens* during abusive cooling regimes in roast beef, (ii) control of *C. perfringens* growth in roast beef using organic acid salts during cooling, (iii) survival of *C. perfringens* vegetative cells and spores during refrigerated storage at 10°C for up to 60 days, and (iv) easy-to-use mathematical models for predicting outgrowth of heat-activated *C. perfringens* spores during cooling of roast beef.

MATERIALS AND METHODS

Bacterial cultures. A three-strain cocktail of enterotoxin-producing *C. perfringens* was used in the study. Strains NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and ATCC 10388 (Hobbs serotype 13) were obtained from Dr. Vijay Juneja (Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, Pa.). The three strains have been implicated in foodborne illness and were selected because of their rapid growth and high heat resistance *(4–6, 13, 16, 29, 33).* Cultures were maintained at 4°C in cooked-meat medium (Difco, Becton Dickinson, Sparks, Md.).

Preparation of spore cocktail. Procedures outlined by Juneja et al. *(14)* were followed for preparation of the spore cocktail. A 0.1-ml aliquot from the stock culture was inoculated into 10 ml of freshly prepared fluid thioglycollate medium (FTM; Difco, Becton Dickinson). Inoculated tubes were heat shocked at 75°C for 20 min in a submerged-coil water bath (Isotemp 3013H, Fisher Scientific, Fair Lawn, N.J.), cooled in chilled water, and incubated at 37°C for 18 h. A 1.0-ml portion of this culture was transferred to 10 ml of freshly steamed FTM and then incubated for 4 h at 37°C. The fresh culture (1%) was then transferred to modified Duncan Strong medium (DS) (10 ml in 1 liter of DS) and incubated aerobically for 24 h at 37°C. The original DS formulation was modified by replacing starch with 0.4% raffinose (Sigma Chemical Co., St. Louis, Mo.) and supplemented with 100 μg/ml of caffeine (Sigma) to enhance sporulation *(14).* DS cultures of each strain were harvested by centrifugation at 7,012 × *g* for 20 min at 4°C (Allegra 21 centrifuge, Beckman, Palo Alto, Calif.) and washed twice in 50 ml of sterile distilled water. The spore crop of each strain was stored separately at 4°C until use. A spore cocktail containing all three strains of *C. perfringens* was prepared immediately before experiments by mixing equivalent numbers of spores from each suspension.

Product. Fat-trimmed beef top rounds from a commercial processor were ground through a 1.0-in. (2.54-cm) grinder plate (Hobart, Troy, Ohio) and mixed at 12% (green weight basis) with brine (marinade) prepared using deionized water to yield a formulated product with 1% salt, 0.2% potato starch (Avebe, Princeton, N.Y.), and 0.2% potassium tetrapyrophosphate (tetrapotassium diphosphate and phosphoric acid solution, Myosol, WTI, Athens, Ga.). The product was reground through a $\frac{1}{16}$-in. (0.16-cm) grinder plate, placed in 250-g bags (3-mil standard barrier nylon vacuum pouch with a water vapor transmission rate of 10 g/liter/m^2/24 h at 37.8°C and 100% relative humidity and an oxygen transmission rate of 3,000 cm^3/liter/m^2/24 h at 23°C and 1 atm; Prime Source, Kansas City, Mo.), vacuum packaged (A300/H, Multivac, Wolfertschweden, Germany), and stored at −20°C until use. Antimicrobial treatments were prepared by thawing the meat overnight at 4.4°C and mixing a portion with antimicrobials to yield the following treatments: 1.3% buffered sodium citrate (BSC; Ional, WTI), 1.3% BSC supplemented with 0.8% sodium diacetate (Ional Plus, WTI), a 2.5% 1:1 mixture of L-sodium lactate (Purasal S, Purac, Lincolnshire, Ill.) and L-potassium lactate (Purasal P, Purac), and a 2.5% 6:4 mixture of L-sodium lactate supplemented with sodium diacetate (Purasal S Opti.form 4, Purac). Meat was mixed in 500-g lots for 1 min with the flat beater accessory on a mixer (Professional 6 model, KitchenAid, St. Joseph, Mich.) in a refrigerated room. Samples were vacuum packaged and stored at −20°C until use.

Sample preparation. Five-gram portions of each thawed treated sample were weighed in a vacuum pouch measuring 2.5 by 5 in. (6.35 by 12.7 cm) (Prime Source). Each subsample was then inoculated with 0.1 ml of the spore cocktail to yield a final concentration of ca. 3.00 log CFU/g of meat. Inoculated samples were vacuum sealed, massaged manually for 30 s to distribute the inoculum homogeneously, flattened to a uniform thickness of ca. 0.2 mm, and stored under refrigeration until use.

Spore activation and cooling profiles. Spores were activated by submerging inoculated bags for 20 minutes into a water bath (Isotemp 3013H) set at 75°C *(18, 19, 31).* Heat shocked samples were then transferred to a refrigerated bath (submerged coil

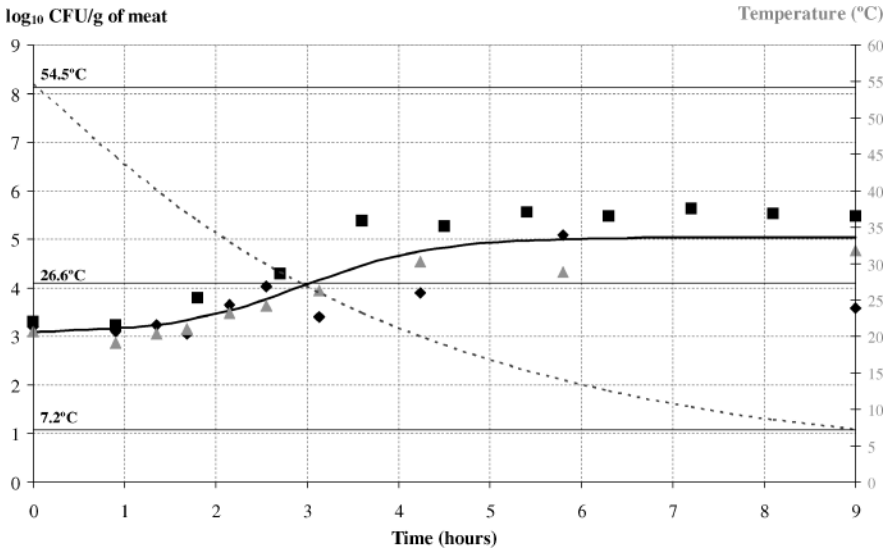

FIGURE 1. *Growth of* C. perfringens *from spores inoculated into vacuum-packaged roast beef samples that were cooled exponentially from 54.5 to 7.2°C in 9 h.* ♦, *replication 1;* ■, *replication 2;* ▲, *replication 3. Solid line is the predicted* C. perfringens *growth; dashed line is the temperature profile.*

apparatus) with water circulation capabilities (RTE 740, Thermo Neslab, Portsmouth, N.H.) that was programmed to cool from 60 to 54.5°C in 5 min. To develop the cooling schedules, core temperature profiles of products were obtained from a roast beef manufacturer located in the Midwest. Commercial profiles and stabilization requirements from the USDA FSIS *(34, 36)* were used to generate cooling curves (Figs. 1 through 5) to cool the product from 54.5 to 4.4°C during the course of 9, 12, 15, 18, or 21 h using models developed by Amézquita et al. *(1)* and Nolan *(22).* Cooling profiles were programmed on the water baths using a commercial software package (NesCom Software, Portsmouth, N.H.). Temperature changes of the cooling processes were simultaneously registered by the water bath thermometer and external data loggers (MKIII model, Temprecord, Modesto, Calif.). Initial spore concentrations from each treatment were evaluated from sample bags immediately after heat shock. Fifteen inoculated samples subjected to heat shock treatment were submerged in the programmable water baths for each trial, and each cooling cycle was run individually. During each exponential cooling period, 10 samples were removed from the water bath at equal intervals. The final samples were removed when the temperature of the bath was 7.2°C. Experimental trials were run in triplicate for each cooling cycle.

Additional bags subjected to heat shocking and cooling for each cycle were removed from the water baths at the end of cooling (7.2°C). Cooled sample bags were submerged in cold water contained in Whirl-Pak bags (Nasco, Modesto, Calif.). Care was taken to assure that samples bags were in contact with the cold water during subsequent storage at 10°C for up to 60 days in a refrigerated incubator. The temperature of the water surrounding the sample bags was continuously monitored with the external data loggers. Individual samples were examined on days 15, 30, 45, and 60 of storage to estimate total *C. perfringens* cells and spore survivors. A total of 225 samples were examined (15 sample bags × five cooling cycles × three replications).

Microbe enumeration. Following removal from the water bath, each sample bag was submerged in alcohol and aseptically opened to transfer the meat to a filter stomacher bag. The meat sample was homogenized with 10 ml of sterile 0.1% buffered peptone water (BPW; Difco, Becton Dickinson) in a Stomacher Lab Blender (Stomacher 400, Seward Medical, London, UK) for 2 min. Serial dilutions were prepared using sterile BPW and then pour plated on tryptose-sulfite-cycloserine agar plates (CM587, Oxoid, Basingstoke, UK) without egg yolk. Solidified plates were overlaid with an additional 5 ml of tryptose-sulfite-cycloserine and incubated for 24 h at 37°C in an anaerobic system (5% CO_2, 10% H_2, and 85% N_2; model 1024, Forma Scientific, Marietta, Ohio).

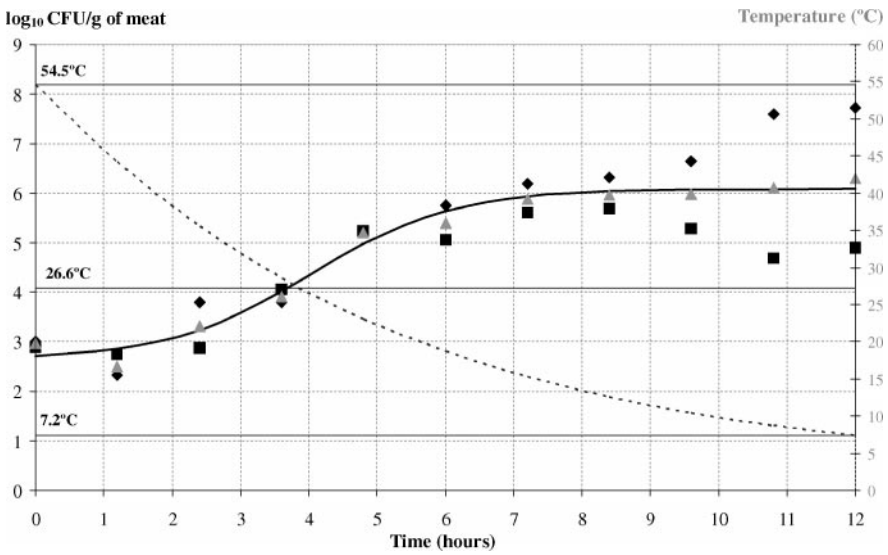

FIGURE 2. *Growth of* C. perfringens *from spores inoculated into vacuum-packaged roast beef samples that were cooled exponentially from 54.5 to 7.2°C in 12 h (see Fig. 1 for descriptions).*

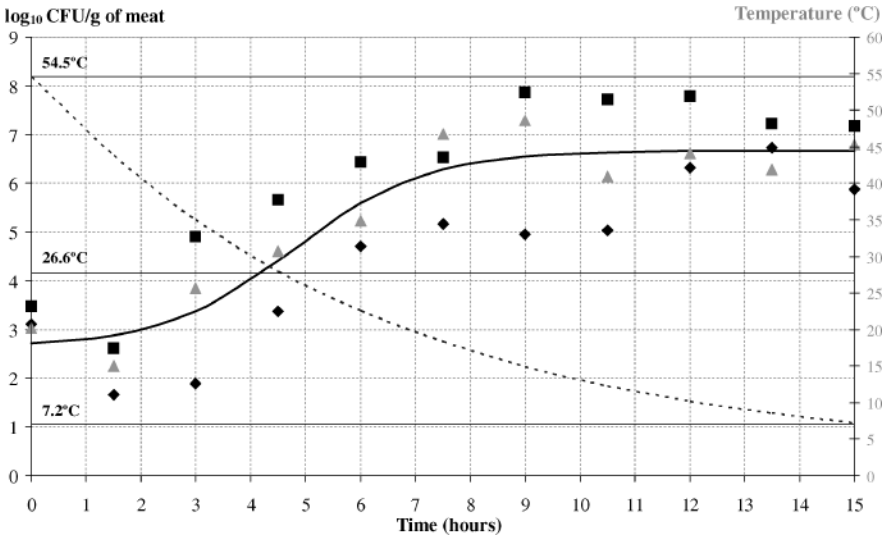

FIGURE 3. *Growth of* C. perfringens *from spores inoculated into vacuum-packaged roast beef samples that were cooled exponentially from 54.5 to 7.2°C in 15 h (see Fig. 1 for descriptions).*

A heat treatment of 15 min at 75°C was used for destruction of vegetative cells and enumeration of *C. perfringens* spore populations in sample homogenates. Total *C. perfringens* populations (vegetative cells and spores) were reported as CFU per gram of sample.

Statistical analysis. Three independent trials were performed for each of the exponential cooling profiles. Experimental data were log transformed, and an analysis of variance (ANOVA) was conducted with the general linear model procedure of SAS (SAS Institute, Cary, N.C.) to evaluate variability between replications for tested intervals during the cooling experiments. The three replications were also analyzed with an ANOVA implemented in the PROC MIXED procedure of SAS to determine differences in the counts during each of the cooling scenarios and during the 10°C storage experiment.

To evaluate growth of *C. perfringens* in roast beef under exponential cooling profiles, experimental growth data were fitted to a four-parameter logistic function (equation 1):

$$y(t) = C + \frac{A}{1 + \exp[-B(t - M)]} \quad (1)$$

where C is the initial inoculum concentration (log CFU per gram), A is the difference between the maximum (higher asymptote) and minimum (lower asymptote) growth values (log CFU per gram), M is the time (hours) at which the slope of the sigmoidal growth curve reaches a maximum value during the exponential phase, and B is the maximum growth rate relative to the amount of growth at time M. The logistic function describes the characteristic bacterial sigmoidal growth curve observed during each of the replications performed under individual cooling profiles.

Each sigmoidal growth curve can be divided into three regions: lag phase (λ), exponential growth phase, and stationary phase. The logistic parameters M, A, and B permit calculation of λ and the exponential growth rate (r_{max}) according to equations 2 and 3:

$$\lambda = M - \frac{1}{B} \quad (2)$$

$$r_{max} = \frac{BA}{4} \quad (3)$$

Data from the three replications were pooled for each cooling rate, and the logistic model (equation 1) was fit separately to the five cooling rate data sets. The models were fit using nonlinear least squares as implemented in SAS PROC NLIN.

RESULTS AND DISCUSSION

Germination and outgrowth under exponential cooling. *C. perfringens* was able to germinate and grow

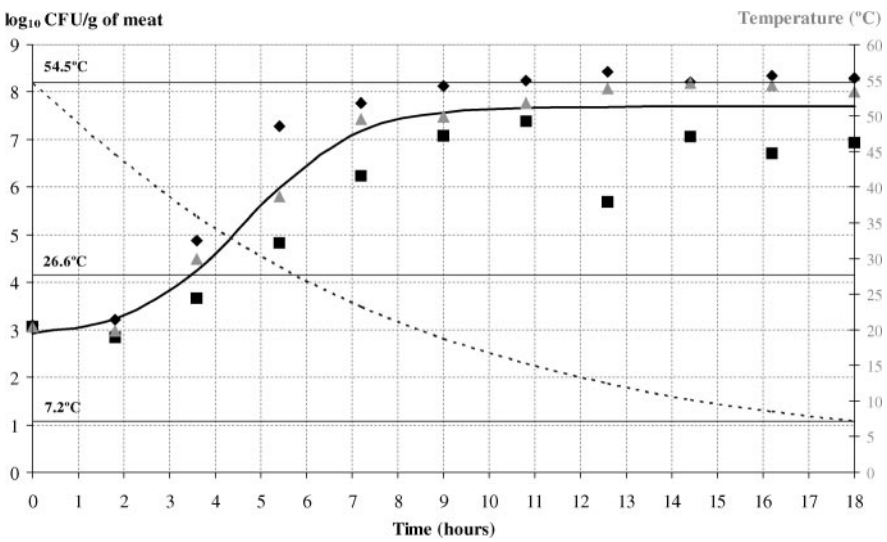

FIGURE 4. *Growth of* C. perfringens *from spores inoculated into vacuum-packaged roast beef samples that were cooled exponentially from 54.5 to 7.2°C in 18 h (see Fig. 1 for descriptions).*

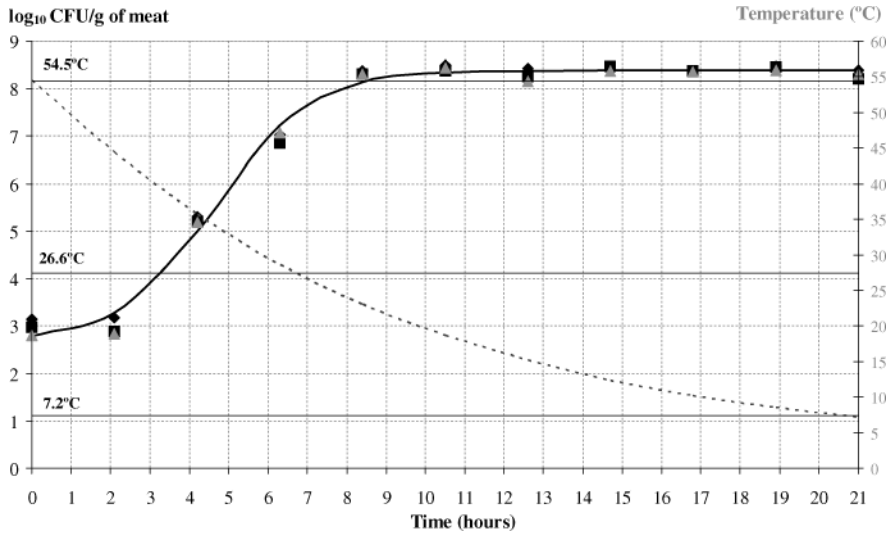

FIGURE 5. *Growth of* C. perfringens *from spores inoculated into vacuum-packaged roast beef samples that were cooled exponentially from 54.5 to 7.2°C in 21 h (see Fig. 1 for descriptions).*

from spores inoculated into commercially formulated roast beef that did not contain any antimicrobials (control). The organism was capable of growing from an initial spore concentration of ca. 3.10 log CFU/g by 2.00, 3.44, 4.04, 4.86, and 5.72 log CFU/g subsequent to 9, 12, 15, 18, and 21 h of exponential cooling, respectively. Growth data from each replication were adjusted to the logistic equation as described by Juneja et al. *(20).* Observed growth data for each replication, adjusted values derived by the logistic function (solid line), and the temperature profiles of each exponential cooling process used in these experiments (dotted line) are depicted in Figures 1 through 5. The estimated parameters for the logistic model are presented in Table 1.

A comparative study was performed to evaluate the effects of the type of heat activation on germination and outgrowth of spores. In a separate experiment, spore-inoculated product was heat activated following product temperature profiles obtained during cooking of roast beef in a commercial processing establishment (Fig. 6). Comparative germination and outgrowth of *C. perfringens* during exponential cooling for 15 h was evaluated either following the cooking profile (commercial process) or the heat shock protocol (20 min at 75 °C). Although the initial spore concentrations were similar between the two heat activation treatments, a reduction in *C. perfringens* population was observed approximately 1 to 1.5 h after start of the cooling process from 54.4 to 7.2°C in the 12- and 15-h cooling regimes, along with an extended lag phase when the product was subjected to the heat shock protocol. The same phenomenon was not observed when the commercial cooking protocol was followed (Fig. 6). The reduction in *C. perfringens* populations may be due to the intensity of the heat shock protocol (75°C for 20 min) compared with a milder treatment, where the spores were subjected to the simulated cooking process. Alternatively, the reduction could be due to sensitization of the germinated spores to various meat product ingredients, such as NaCl and/or phosphates, during heat shock.

TABLE 1. *Estimated parameters for the logistic model described in equation 4 for prediction of* C. perfringens *growth in roast beef cooled exponentially from 54.5 to 7.2°C over the course of 9, 12, 15, 18, or 21 h*[a]

cr (h)	*C* (log CFU/g)	*A* (log CFU/g)	*B* (h)	*M* (h)	σ^2	λ (h)	r_{max} (1/h)
9	3.07	1.98	1.41	2.99	0.28	2.28	0.70
12	2.64	3.44	0.97	4.02	0.40	2.99	0.83
15	2.64	4.04	0.83	4.79	0.87	3.59	0.84
18	2.84	4.86	0.85	4.67	0.51	3.49	1.03
21	2.67	5.72	0.83	4.67	0.04	3.47	1.19

[a] *cr*, cooling rate; *C*, initial concentration of inoculum; *A*, difference between maximum and minimum growth values; *M*, time at which the slope of the sigmoidal growth reaches a maximum value; *B*, maximum growth rate relative to the amount of growth at time *M*; σ^2, square error of the estimates. Estimates were obtained with the PROC NLIN precedure of SAS and were used to calculate the lag phase duration (λ) and the exponential growth rate (r_{max}) using equations 2 and 3.

The exposure of bacterial spores to excessive heating can cause injury *(8).* Johnson and Busta *(12)* reported that initial heat shock treatments can induce subsequent temperature sensitivity in *Bacillus cereus* during outgrowth from spore inocula during exponential cooling and at static temperatures (45°C). The heat shock protocol used in most of the previous studies evaluating *C. perfringens* spore germination and outgrowth *(9, 10, 15, 19)* may have triggered the cells to enter an injury-repair state known as the Phoenix phenomenon *(25).* The effects of this phenomenon may directly influence biokinetic parameters (lag phase duration and exponential growth rates) of *C. perfringens* and may have influenced the performance of predictive models developed following the heat shock protocol for activation of the spores.

Exponential cooling of roast beef from 54.5 to 7.2°C in 9 h resulted in *C. perfringens* growth of ca. 2.00 log CFU/g. The lag-phase duration and exponential growth rate estimated by the logistic function were 2.3 h and 0.70 log CFU/g/h, respectively. Shigehisa et al. *(24)* observed ca. 3.00 log CFU/g growth in *C. perfringens* in autoclaved ground beef cooled exponentially from 60 to 15°C for 9 h

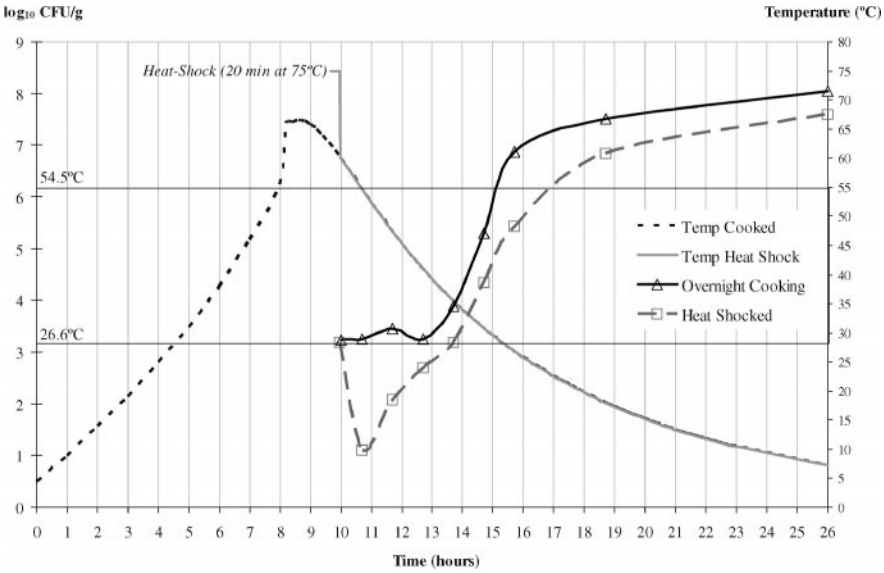

FIGURE 6. *Growth comparison of* C. perfringens *spores inoculated into vacuum-packaged roast beef samples and activated by the heat shock protocol (20 min at 75°C). A simulated commercial cooking profile (□) was followed by exponential cooling from 54.5 to 7.2 °C in 15 h (▲).*

with an observed lag phase of 4 h. However, Steele et al. *(29)* reported only 0.70 and 1.30 log CFU/g growth in turkey roast cooled from 48.9 to 12.8°C in 8 and 10 h, respectively. Huang *(9)* evaluated the outgrowth of *C. perfringens* spores in cooked ground beef during linear cooling from 51 to 10°C in 7 h and observed germination and outgrowth by ca. 2.00 log CFU/g. These differences in *C. perfringens* growth and biokinetic parameters (lag-phase duration and exponential growth rate) could be due to differences in the product formulations and the meat product characteristics such as pH and water activity. In the present study, the product formulation included NaCl and phosphate, which are common ingredients in roast beef and other processed meat formulations used in the industry. The meat formulation used represented a worst-case scenario because the amounts of these ingredients were slightly lower than those used commercially, thus providing a conservative estimate of *C. perfringens* spore germination and outgrowth potential.

The Pathogen Modeling Program (version 6.1) from the USDA Agricultural Research Service (ARS) estimated a ca. 0.50 log CFU/ml outgrowth of *C. perfringens* spores in beef broth during exponential cooling from 54.5 to 7.2°C in 9 h. The difference indicates that the Pathogen Modeling Program model available for beef broth may underestimate the growth that occurs in a meat sample, thus limiting its applicability in commercial meat products. These underestimations of *C. perfringens* growth may be due to the use of laboratory media in developing the model; in some studies *C. perfringens* has grown at a faster rate in meat samples than in laboratory media *(37)*. The limitations of this model have been reported in a recent publication in which *C. perfringens* growth estimations from predictive model were compared with growth in a meat product *(28)*. The authors reported that the USDA ARS model performs relatively well (fail safe) when low (<1 log CFU/ml) or high (>3 log CFU/ml) *C. perfringens* population increases occur during exponential cooling. However, the model consistently underpredicted growth at intermediate *C. perfringens* population increases (1 to 3 log CFU/ml) and in trials using two different rates of exponential cooling.

Exponential cooling of roast beef from 54.5 to 7.2°C in 12 h resulted in *C. perfringens* growth of 3.44 log CFU/

TABLE 2. *Parameters, estimates, and error for the predicted model from equation 4*

Parameter[a]	Estimate[b]	Error	$P > \|t\|$
C	2.6718	0.3709	<0.0001
*C*1	0.3938	0.4791	0.4123
*C*2	−0.0282	0.5110	0.9561
*C*3	−0.0358	0.4933	0.9422
*C*4	0.1698	0.5210	0.7449
A	5.7185	0.4113	<0.0001
*A*1	−3.7415	0.5717	<0.0001
*A*2	−2.2771	0.6061	0.0002
*A*3	−1.6838	0.5672	0.0034
*A*4	−0.8623	0.5854	0.1427
B	0.8303	0.1755	<0.0001
*B*1	0.5823	0.7669	0.4488
*B*2	0.1382	0.4163	0.7404
*B*3	−0.0001	0.2846	0.9998
*B*4	0.0170	0.2790	0.9516
M	4.6598	0.3344	<0.0001
*M*1	−1.6746	0.5821	0.0046
*M*2	−0.6378	0.5243	0.2256
*M*3	0.1315	0.5326	0.8053
*M*4	0.0127	0.4993	0.9798

[a] *C*, initial concentration of inoculum; *A*, difference between maximum and minimum growth values; *M*, time at which the slope of the sigmoidal growth reached a maximum value; *B*, maximum growth rate relative to the amount of growth at time *M* for the 21-h cooling cycle. These values were used as the baseline to calculate other estimates. Numbers 1 through 4 are adjustments to the baseline values based on the cooling rate (1, 9 h of cooling; 2, 12 h; 3, 15 h; 4, 18 h).

[b] These estimated values are used in the equation to predict (*P*) the log CFU per gram of sample at a time *t* when roast beef is subjected to cooling from 54.5 to 7.2°C in 9, 12, 15, 18, or 21 h.

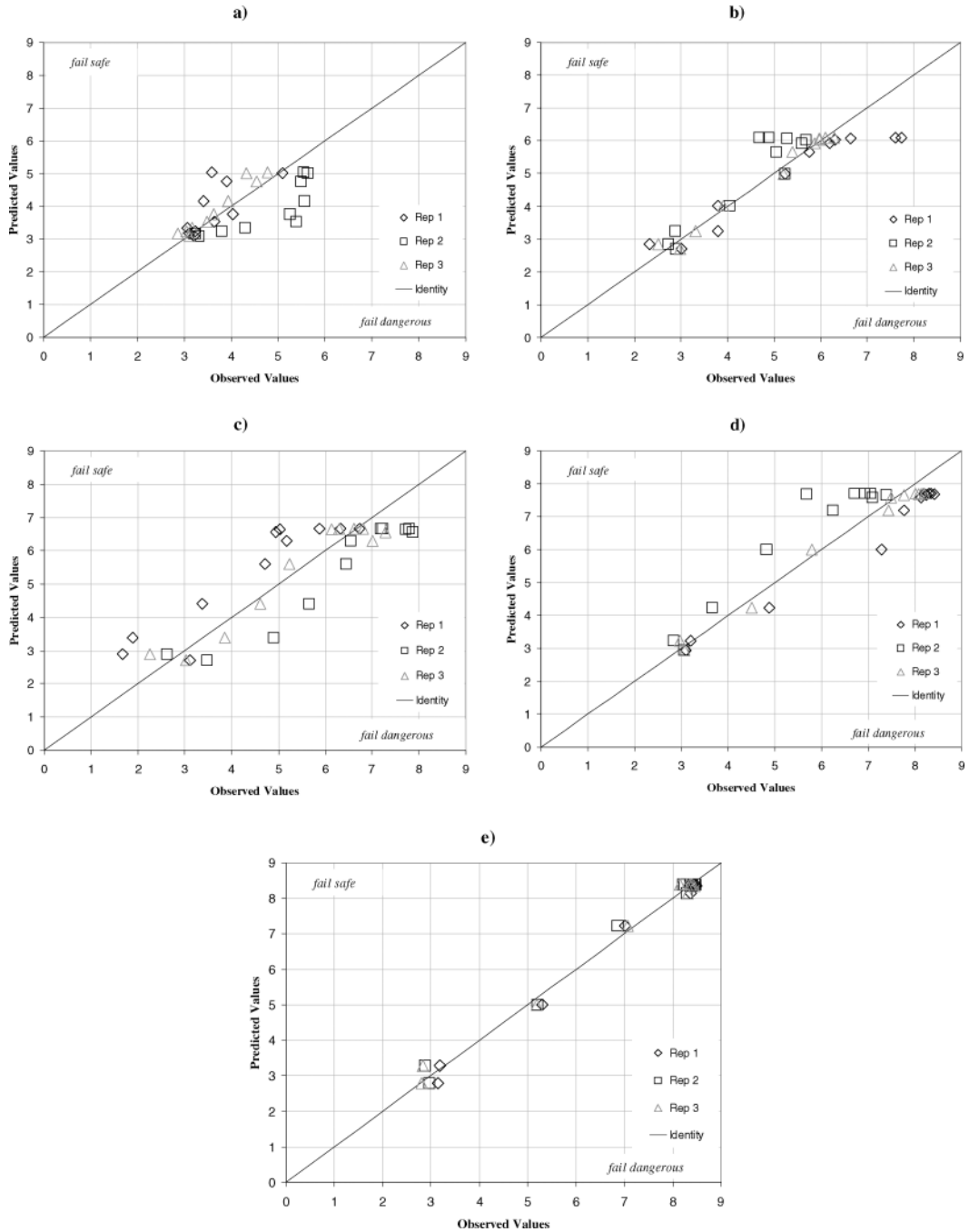

FIGURE 7. *Performance evaluation of the model comparing predicted and observed growth of* C. perfringens *in roast beef samples cooled exponentially from 54.5 to 7.2 °C in (a) 9, (b) 12, (c) 15, (d) 18, and (e) 21 h.*

g. A lag-phase duration of ca. 3 h and exponential growth rate of 0.83 log CFU/g/h were observed. These results differ significantly from the findings of Juneja et al. *(18)*, who reported no growth during exponential cooling from 54.5 to 7.2°C for 12 h. These differences may be due to lower inoculation concentrations (1.50 log CFU/g) or lack of proper temperature control of the product; ice was manually added at intervals to adjust the water bath temperature and consequently the product temperature. Other potential source of variation between the two studies, resulting in differences in *C. perfringens* germination and outgrowth, could be the type of packaging film (Whirl-Pak bags versus commercial meat packaging film) *(18)*. Smith et al. *(27)* reported that *C. perfringens* growth was affected by the packaging film used; lower growth occurred in ground beef packaged in Whirl-Pak bags because of the greater oxygen permeability.

Extending the exponential cooling (from 54.5 to 7.2°C) period to 15 h resulted in a 4.04 log CFU/g increase in *C. perfringens* populations, with a lag-phase duration of 3.6 h and an exponential growth rate of 0.84 log CFU/g/h. These results are similar to those observed by Juneja and Thippareddi *(19)* in ground turkey breast meat. The authors reported *C. perfringens* growth by ca. 3.80 log CFU/g during a 15-h cooling profile, similar to the one used in this experiment. Similarly, Zaika *(38)* reported *C. perfringens*

TABLE 3. C. perfringens *total cell counts and spore counts in heat shocked roast beef subjected to cooling from 54.5 to 7.2°C in 9 h and stored for 15, 30, 45, and 60 days at 10°C*[a]

	Total cells (log CFU/g)					Spores (log CFU/g)				
Time (h)	C	BSC	BSC+D	L	L+D	C	BSC	BSC+D	L	L+D
0	3.32 A	3.02 A	2.87 A	3.08 A	3.20 A	3.32 A	3.02 A	2.87 A	3.08 A	3.20 A
0.95	1.75 AC	2.74 A	2.64 A	2.77 A	ND C	ND C	1.74 AC	1.69 AC	2.20 A	1.93 AC
4.68	3.00 A	3.34 A	2.61 A	2.78 A	2.07 A	ND C	1.59 AC	1.99 AC	2.36 A	1.30 AC
9	5.04 B	2.79 A	2.75 A	2.45 A	1.93 AC	ND C	1.67 AC	2.20 A	1.07 AC	1.29 AC
360	1.57 AC	0.49 C	0.29 C	0.38 C	0.31 C	0.23 C	1.62 AC	2.09 A	1.43 AC	0.93 C
720	1.16 AC	ND C	ND C	0.23 C	ND C	ND C	1.40 AC	2.17 A	1.39 AC	0.76 C
1,080	ND C	ND C	ND C	ND C	ND C	ND C	1.20 AC	2.20 A	1.49 AC	0.28 C
1,440	ND C	ND C	ND C	ND C	ND C	ND C	0.96 C	1.93 AC	1.58 AC	0.62 C

[a] Treatments: C, control; BSC, buffered sodium citrate (1.3%); BSC+D, BSC plus sodium diacetate; L, sodium plus potassium lactates (1:1); L+D, sodium lactate plus sodium diacetate (6:4). ND, nondetectable levels. Values are means of three replications. Values followed by the same letters are not significantly different ($P \geq 0.05$).

growth of ca. 3.00 log CFU/g of ground beef, but growth was inhibited (0.72 log CFU/g) when the salt concentration was increased to 2% and growth was prevented at salt concentrations ≥3%

Extension of exponential chilling (from 54.5 to 7.2°C) time to ≥9 h resulted in >1.00 log CFU/g growth of *C. perfringens* under anaerobic conditions in roast beef containing no antimicrobial ingredients.

Growth of *C. perfringens* was evaluated during extended cooling of noncured roast beef treated with 1.3% BSC, a 1.3% mixture of BSC and sodium diacetate, a 2.5% mixture (1:1) of sodium and potassium lactates, and a 2.5% mixture of sodium lactate and sodium diacetate. Growth was not observed in any of the samples containing the antimicrobials, indicating that the antimicrobial concentrations were sufficient to inhibit germination and/or outgrowth of activated spores under extended cooling conditions. Addition of these antimicrobial agents into noncured products permits the extension of cooling to up to 21 h with no observed growth of *C. perfringens* under conditions of deviation from standard cooling protocols. Sodium lactate is effective at inhibiting the growth of *C. perfringens* in beef when formulated at concentrations above 3% *(3)*. Juneja and Thippareddi *(19)* and Thippareddi et al. *(31)* reported that sodium lactate was effective at inhibiting the growth of *C. perfringens* when used at concentrations higher than 2%. These authors reported inhibition of *C. perfringens* growth following exponential cooling from 54.5 to 7.2°C in 18 or 21 h by BSC and BSC supplemented with sodium diacetate at lower concentrations (≥1%) than those of sodium lactate (≥2%) in roast beef and ground, injected pork. Similar results were reported by Sabah et al. *(23)*, who evaluated sodium citrate buffered to different pH values and at concentrations ranging from 2 to 4.8% in meat and found no differences in inhibition at different BSC pH values. This lack of difference could be due to the ability of the BSC to completely inhibit *C. perfringens* germination and/or outgrowth at lower concentrations (ca. 1.0%), resulting in no *C. perfringens* growth at any of the concentrations evaluated in the study.

Predictive model. The majority of the predictive models describing growth of microorganisms in foods have been developed using static temperature conditions to develop primary models as a function of time. These models are then expressed as a function of temperature, a process known as secondary modeling. A model was developed using the logistic function to describe sigmoidal *C. perfrin-*

TABLE 4. C. perfringens *total cell counts and spore counts in heat shocked roast beef subjected to cooling from 54.5 to 7.2°C in 12 h and stored for 15, 30, 45, and 60 days at 10°C*[a]

	Total cells (log CFU/g)					Spores (log CFU/g)				
Time (h)	C	BSC	BSC+D	L	L+D	C	BSC	BSC+D	L	L+D
0	2.96 A	3.00 A	2.93 A	3.11 A	3.28 A	2.96 A	3.00 A	2.93 A	3.11 A	3.28 A
1.2	2.53 A	2.63 A	2.57 A	2.17 A	2.30 A	0.23 C	1.85 AC	1.54 AC	1.44 AC	1.77 AC
6	5.40 B	2.67 A	2.46 A	2.41 A	1.83 AC	ND C	1.79 AC	1.30 AC	1.34 AC	0.66 C
12	6.13 B	2.46 A	2.47 A	2.09 A	1.29 AC	ND C	1.38 AC	1.22 AC	1.33 AC	0.69 C
360	3.29 A	ND C	ND C	1.00 AC	ND C	ND C	1.28 AC	1.35 AC	1.28 AC	0.82 C
720	3.12 A	ND C	ND C	0.23 C	ND C	ND C	1.03 AC	1.43 AC	1.13 AC	0.93 C
1,080	2.57 A	0.23 C	0.23 C	ND C	ND C	ND C	1.25 AC	1.04 AC	1.16 AC	0.42 C
1,440	2.24 A	ND C	ND C	ND C	ND C	ND C	1.09 AC	0.98 C	0.36 C	ND C

[a] ND, nondetectable levels. Values are means of three replications. Values followed by the same letters are not significantly different ($P \geq 0.05$. See Table 3 for treatment descriptions.

TABLE 5. C. perfringens *total cell counts and spore counts in heat shocked roast beef subjected to cooling from 54.5 to 7.2°C in 15 h and stored for 15, 30, 45, and 60 days at 10°C*[a]

	Total cells (log CFU/g)					Spores (log CFU/g)				
Time (h)	C	BSC	BSC+D	L	L+D	C	BSC	BSC+D	L	L+D
0	3.21 A	2.81 A	3.04 A	3.02 A	3.21 A	3.21 A	2.81 A	3.04 A	3.02 A	3.21 A
1.5	2.18 A	2.10 A	2.24 A	2.64 A	2.52 A	0.23 C	2.45 A	2.66 A	2.52 A	2.14 A
7	6.24 B	2.39 A	1.54 AC	2.40 A	2.26 A	0.74 C	2.77 A	2.24 A	1.98 AC	1.59 AC
15	6.62 B	2.30 A	1.85 AC	2.49 A	1.69 AC	0.44 C	2.48 A	1.66 AC	2.49 A	1.01 AC
360	1.79 AC	0.23 C	0.34 C	0.54 C	0.28 C	0.49 C	2.20 A	2.60 AC	1.51 AC	0.99 C
720	2.48 A	ND C	ND C	0.54 C	ND C	ND C	2.27 A	2.27 A	2.37 A	1.91 AC
1,080	1.86 AC	ND C	0.23 C	ND C	ND C	ND C	1.79 AC	2.20 A	1.84 AC	1.80 AC
1,440	1.25 AC	ND C	ND C	ND C	ND C	0.31 C	1.91 AC	2.41 A	2.44 A	1.71 AC

[a] ND, nondetectable levels. Values are means of three replications. Values followed by the same letters are not significantly different ($P \geq 0.05$. See Table 3 for treatment descriptions.

gens growth curves for outgrowth during cooling from 54.5 to 7.2°C within 9, 12, 15, 18, and 21 h.

To accommodate both time and cooling rate in the model, the logistic function (equation 1) was expanded to include *C*, *A*, *B*, and *M* parameters for each cooling rate:

$$P = (C_0 + C_1cr_1 + C_2cr_2 + C_3cr_3 + C_4cr_4) + \{(A_0 + A_1cr_1 + A_2cr_2 + A_3cr_3 + A_4cr_4) \div [1 + \exp\{[-(B_0 + B_1cr_1 + B_2cr_2 + B_3cr_3 + B_4cr_4)] \times [t - (M_0 + M_1cr_1 + M_2cr_2 + M_3cr_3 + M_4cr_4)]\}]\} \quad (4)$$

where P is the predicted growth of *C. perfringens.* The parameters C_0, A_0, B_0, and M_0 are the baseline parameters, and they correspond to the 21-h cooling rate. The variables cr_1 through cr_4 are scored as either 0 or 1, indicating whether the observation is from a particular cooling rate. For example, if an observation is from the 9-h cooling rate, then $cr_1 = 1$; otherwise, $cr_1 = 0$. The variables cr_2, cr_3, and cr_4 correspond to cooling rates 12, 15, and 18 h, respectively. The parameters C_i, A_i, B_i, and M_i (where $i = 1, 2, 3,$ or 4) represent adjustments to the baseline values based on the cooling rate. For example, the initial inoculation concentration (log CFU per gram) for the cooling rate of 15 h is given by $C_0 + C_3$.

The augmented logistic model was fit to the data pooled over all replications and cooling rates. Model fitting was carried out using SAS PROC NLMIXED because it allows the user to compare functions of the parameter estimates. The estimated logistic parameters from the individual cooling rates (Table 1) were used as starting values in PROC NLMIXED. The estimated parameters for the augmented logistic model are given in Table 2.

Performance of the model was evaluated by plotting growth data predicted by the model versus observed data obtained from the validation. *C. perfringens* growth during exponential cooling for 9, 12, 15, 18, and 21 h was plotted versus the predictions derived with equation 4 in Figure 7a through 7e, respectively. Data points on the solid line (line of identity) indicate accuracy of predictions. Data points in the fail-safe region indicate that the model overpredicted actual growth of *C. perfringens.* In contrast, values in the fail-dangerous region indicate that the model underpredicted *C. perfringens* growth. In general, the model developed performed well in predicting *C. perfringens* growth during exponential cooling of roast beef.

This simple approach permits the user to predict growth of an organism under the cooling conditions specified. Although the model will not allow interpolation between cooling rates, approximation of growth characteristics can assist processors in making decisions for any deviation in the standard cooling protocol. Ideally, the processor must have followed a cooling profile that matches the temperature conditions presented in this study. Because the cooling profiles were developed using commercial processing data and heat transfer considerations, a majority of cooling profiles should closely match the temperature regimes presented in this study. When cooling profiles do not fall in the ranges covered in this study, approximations could be made by using the faster and slower cooling protocols to run the model. However, the information provided cannot be extrapolated to other types of meat products because changes in the formulation of the product or in the meat species (pork or poultry meat) will influence the growth characteristics of *C. perfringens* during cooling. An easy-to-use Microsoft Excel worksheet can be constructed with the model to provide valuable information to meat processors.

Fate of *C. perfringens* spores and vegetative cells under abusive storage conditions (10°C). Survival and destruction of total cell and spore populations of *C. perfringens* inoculated into roast beef formulated with and without antimicrobials were evaluated (Tables 3 through 7). Prolonged storage reduced vegetative *C. perfringens* populations. These populations decreased consistently in control samples, which allowed *C. perfringens* spore germination and outgrowth during cooling from an initial population of ca. 3.10 log CFU/g, especially in sample bags subjected to longer cooling scenarios (18 and 21 h).

Reductions in *C. perfringens* populations of 3.47, 3.02,

TABLE 6. C. perfringens *total cell counts and spore counts in heat shocked roast beef subjected to cooling from 54.5 to 7.2°C in 18 h and stored for 15, 30, 45, and 60 days at 10°C*[a]

	Total cells (log CFU/g)					Spores (log CFU/g)				
Time (h)	C	BSC	BSC+D	L	L+D	C	BSC	BSC+D	L	L+D
0	3.07 A	3.01 A	2.99 A	3.09 A	3.06 A	3.07 A	3.01 A	2.99 A	3.09 A	3.06 A
1.8	3.01 A	2.76 A	2.81 A	2.78 A	2.58 A	1.01 AC	2.35 A	2.85 A	2.99 A	2.52 A
9	7.57 B	2.33 A	2.02 A	2.55 A	1.74 AC	ND C	2.51 A	2.74 A	2.77 A	1.94 AC
18	7.75 B	2.14 A	2.40 A	2.22 A	1.56 AC	0.41 C	2.41 A	2.85 A	2.79 A	1.87 AC
360	4.53 AB	ND C	ND C	ND C	ND C	ND C	2.33 A	2.61 A	2.38 A	1.78 AC
720	1.88 AC	ND C	ND C	ND C	ND C	ND C	2.64 A	2.67 A	2.70 A	0.71 C
1,080	0.77 AC	ND C	ND C	ND C	ND C	ND C	2.57 A	2.44 A	2.67 A	2.38 A
1,440	1.07 AC	ND C	0.23 C	ND C	ND C	ND C	2.29 A	2.51 A	2.53 A	1.40 AC

[a] ND, nondetectable levels. Values are means of three replications. Values followed by the same letters are not significantly different ($P \geq 0.05$. See Table 3 for treatment descriptions.

4.14, 3.22, and 4.12 log CFU/g (in roast beef chilled from 54.4 to 7.2°C within 9, 12, 15, 18 and 21 h, respectively) were observed after 15 days of storage at 10°C. Additional *C. perfringens* reductions were observed during refrigerated storage beyond 15 days, as reported by Kalinowski et al. *(21)* and Taormina et al. *(30)*. Kalinowski et al. observed reductions in *C. perfringens* of 2.52, 2.54, and 2.75 log CFU/g in cured turkey held at 0.6, 4.4, and 10°C for 7 days, whereas Taormina et al. reported less dramatic reductions (1.97 log CFU/g) in *C. perfringens* populations after 14 days of storage at 4.4°C in chicken bologna.

Minimal or nondetectable *C. perfringens* spore populations were observed after only a few hours of cooling and during subsequent refrigerated storage. This finding indicates that most of the spores inoculated into roast beef were capable of germination and outgrowth into vegetative cells during the cooling process.

Incorporation of antimicrobials (salts of organic acids) resulted in reduction in total *C. perfringens* populations (Tables 3 through 7). Minimal to nondetectable levels of total *C. perfringens* cells were observed in roast beef containing 1.3% BSC, a 1.3% mixture of BSC and sodium diacetate, a 2.5% mixture (1:1) of sodium and potassium lactates, and a 2.5% mixture of sodium lactate and sodium diacetate during storage at 10°C. However, *C. perfringens* spore populations in roast beef containing these antimicrobials remained relatively stable after prolonged storage at 10°C, with minimal reductions (1.00 to 1.50 log CFU/g) observed after refrigerated storage for 60 days. Persistence of *C. perfringens* spores in roast beef containing the antimicrobials may be due to prevention of germination of a significant proportion of the spore population. *C. perfringens* spore populations were not observed in roast beef that did not contain the antimicrobials, indicating that the majority of spores germinated and grew after heat treatment or thermal processing.

Prevention of spore germination in product containing antimicrobials may result in a new food safety hazard in products that do not contain the antimicrobials and that have been prepared using the meat products that do contain them (e.g., chili and soups). Nongerminating spores that were inhibited by the antimicrobials may subsequently germinate and grow to hazardous levels when meat samples are improperly handled or these products are used as ingredients in other prepared meals such as soups and gravies. Meat products used in food service operations could be cooked with the addition of gravies and other ingredients that may render the concentration of the formulated antimicrobials insufficient to prevent *C. perfringens* spore germination. Reheating and improper holding of these prod-

TABLE 7. C. perfringens *total cell counts and spore counts in heat shocked roast beef subjected to cooling from 54.5 to 7.2°C in 21 h and stored for 15, 30, 45, and 60 days at 10°C*[a]

	Total cells (log CFU/g)					Spores (log CFU/g)				
Time (h)	C	BSC	BSC+D	L	L+D	C	BSC	BSC+D	L	L+D
0	2.97 A	2.93 A	3.10 A	3.17 A	3.30 A	2.97 A	2.93 A	3.10 A	3.17 A	3.30 A
2.1	2.97 A	2.21 A	2.26 A	2.22 A	1.65 AC	ND C	2.90 A	2.94 A	2.73 A	1.82 AC
10.5	8.44 B	1.64 AC	1.59 AC	2.17 A	1.46 AC	ND C	2.12 A	2.76 A	2.23 A	2.05 A
21	8.30 B	1.79 AC	1.90 AC	2.38 A	1.30 AC	ND C	2.80 A	2.62 A	1.34 A	1.69 AC
360	4.18 AB	ND C	ND C	ND C	ND C	ND C	1.67 AC	2.28 A	2.22 A	1.73 AC
720	3.10 A	0.18 C	0.36 C	ND C	ND C	ND C	2.28 A	2.69 A	2.58 A	1.44 AC
1,080	1.45 AC	ND C	ND C	0.23 C	ND C	ND C	2.02 A	2.11 A	2.09 A	1.46 AC
1,440	0.60 AC	ND C	ND C	ND C	ND C	ND C	1.92 AC	2.13 A	2.16 A	1.32 AC

[a] ND, nondetectable levels. Values are means of three replications. Values followed by the same letters are not significantly different ($P \leq 0.05$. See Table 3 for treatment descriptions.

ucts (soups and gravies) may allow *C. perfringens* spores to become activated, to germinate, and to grow to hazardous levels. Therefore, it is important that food service operations follow good manufacturing practices to avoid these potentially hazardous situations.

Addition of GRAS antimicrobials to processed meats can provide a safety margin to prevent germination and outgrowth of *C. perfringens* from spores during extended cooling periods or cooling process deviations. Predictive models can be used to evaluate the safety of cooling regimes for processed meat or of product when deviations from safe harbors occur during processing. However, caution should be exercised when applying predictions from models developed with different product characteristics, such as meat species, pH, and ingredients such as nitrite, phosphates, salt, and other additives.

ACKNOWLEDGMENT

This project was supported by the Cooperative State Research Education and Extension Service, USDA, under agreement 25-6231-0078-001.

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